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Reactivation of Breast Cancer Micrometastases by Senescent Bone Marrow Stroma

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14. ABSTRACT We developed an injury model to bone marrow stroma cultured in vitro from bone marrow samples from mice and human donors. We demonstrated that oxidative injury with H ₂ O ₂ induces a reproducible time- and dose-dependent secretory senescence profile that consists of export of interleukin 6 (IL-6) by murine and human stroma and IL-8 by murine stroma and activation of TGFβ signaling. Using oxidative injury as a positive control, we demonstrated that estrogen deprivation induces secretory senescence in bone marrow stroma both in female mice and a premenopausal human female volunteer and activation of TGFβ signaling in mice. We defined the parameters for estrogen-induced secretory senescence in mouse and human marrow. Preliminary observations suggest that human marrow is more sensitive to estrogen deprivation-induced secretory senescence resulting in a greater and more sustained response. These data will be expanded to define the relationship between post menopausal status and secretory senescence and the effect on this stromal response on the capacity to support micrometastatic breast cancer cell dormancy.					
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INTRODUCTION:

The proposed project investigates one potential mechanism for the reawakening of microscopic breast cancer metastases in the bone marrow of patients at significant periods of time after initial treatment for local disease. The hypothesis being tested is that deprivation of estrogen that occurs after menopause may be a contributing factor to the generation of senile senescence in the bone marrow stroma of women. In this model, inflammatory cytokines generated by secretory senescent stromal cells induce dormant breast cancer cells to re-enter the cell cycle and grow into recurrent tumors in the bone. We began testing this hypothesis in the first year by defining the secretory senescence phenotype initially in murine stroma and confirming it in human stroma, using hypoxic and oxidative stress, and subsequently using estrogen deprivation.

BODY:

Initial studies were conducted in murine stroma to characterize the system and the reagents, since human stromal samples are less easily accessible than those from mice. We developed a stromal culture model from murine bone marrow obtained from female Ncr nu/nu mice sacrificed for other projects, some by other labs that were going to be discarded. Bone marrow was cultured on 25 cm² flasks in Gartner's Medium until a stromal layer formed at the bottom of the flask. Stromal cells were trypsinized and distributed to 24 well plates and cultured until confluent.

We first determined the effects of the timing and dosage of hydrogen peroxide (H₂O₂), an agent that generates oxidative stress and of carbonyl-cyanide m-chlorophenylhydrazone (CCCP) an agent that blocks the mitochondrial electron transport chain, to create hypoxic stress, on injury to stroma as determined by lactate dehydrogenase (LDH) release. Figure 1 demonstrates the effects of 10, 100 and 1,000 μM H₂O₂ and Figure 2 the effects of 10, 100 and 1,000 μM CCCP on the relative increase in LDH in the media at 24, 48, 72 hours and six days after a one hour incubation. Cytotoxicity was achieved by 24 hours with both 100 μM H₂O₂ and CCCP by 24 hours but the effects of the injury began to ebb by 3 to 7 days, probably because the injured cells had died already. These experiments were done to design positive control conditions for stromal injury.

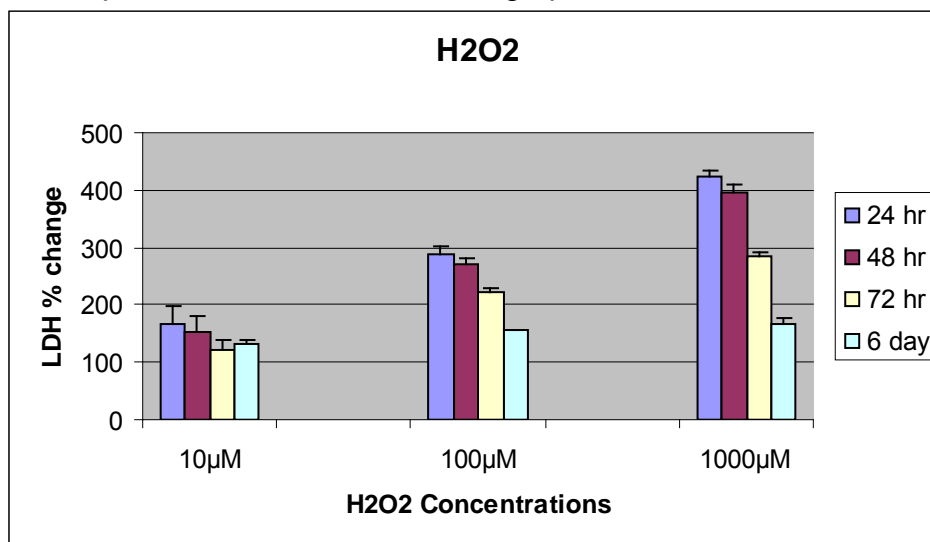


Figure 1. Time and dose effects of H₂O₂ on LDH release by murine stromal cells. Near confluent stromal monolayers cultured in 24-well plates were treated with H₂O₂ for 1 hour and then washed once with PBS. Cells were then incubated in conditioned medium with only 5% FBS. Lactate dehydrogenase (LDH) release was measured at 24h, 48h, 72h, and 6 days using the Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega) following the manufacturer's instructions. Data represent % LDH change of the test group compared to that of the control group. *Columns*: means; *bars*: SE. Experiments were done in triplicate at four samples per point. Data are from one representative experiment.

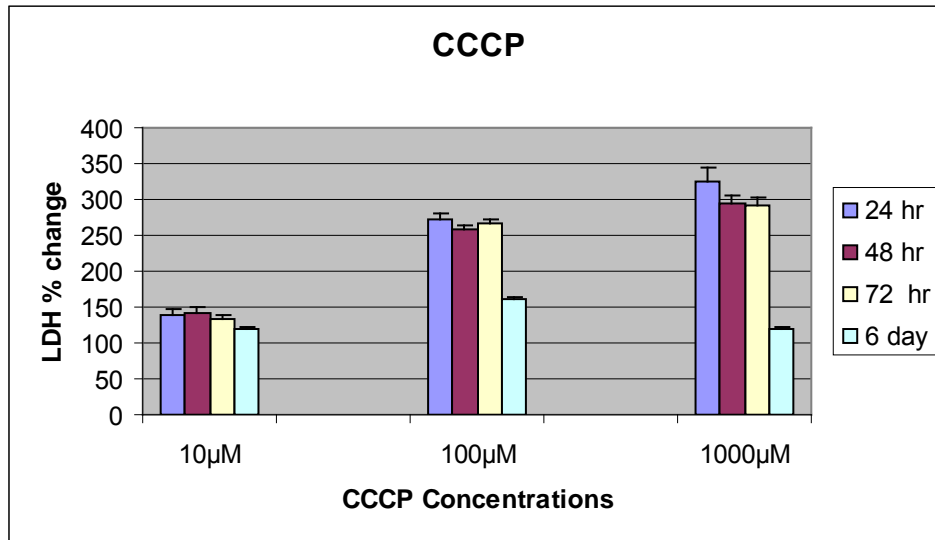


Figure 2. Time and dose effects of CCCP on LDH release by murine stromal cells. Near confluent stromal monolayers cultured in 24-well plates were treated with CCCP for 1 hour and then washed once with PBS. Cells were then incubated in conditioned medium with only 5% FBS. Lactate dehydrogenase (LDH) release was measured at 24h, 48h, 72h, and 6 days using the Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega) following the manufacturer's instructions. Data represent % LDH change of the test group compared to that of the control group. *Columns*: means; *bars*: SE. Experiments were done in triplicate at four samples per point. Data are from one representative experiment.

The next series of experiments were designed to develop an ELISA assay for export of murine IL-6 and murine IL-8 (KC) as representative assays for secretory senescence in stroma as a consequence of treatment with H₂O₂ and CCCP. We determined the effects of the timing and dosage of H₂O₂ and CCCP on the presence of IL-6 and IL-8 (KC). Figure 3 demonstrates the effects of 10, 100 and 1,000 μM H₂O₂ and Figure 4 the effects of 10, 100 and 1,000 μM CCCP on the concentration of IL-6 in the media at 24, 48, 72 hours and six days after a one hour incubation. Similarly, Figures 5 and 6 reflect the effects of the same treatments on the concentration of IL-8 (KC) in the same experiments.

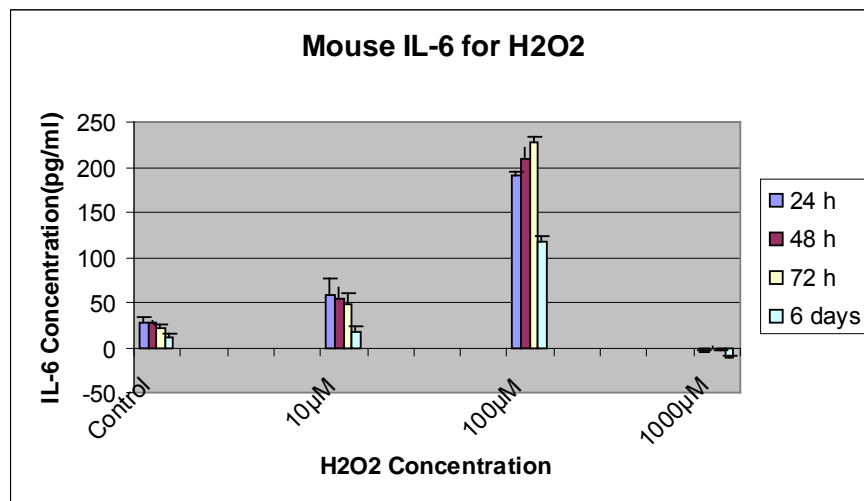


Figure 3. Time and dose effects of H₂O₂ on IL-6 in the media of murine stromal cells. Near confluent stromal monolayers cultured in 24-well plates were treated with H₂O₂ for 1 hour and then washed once with PBS. Cells were then incubated in conditioned medium with only 5% FBS. Supernatant samples were collected at 24h, 48h, 72 hours and 6 days. The IL-6 levels in the supernatant were determined using an IL-6 ELISA kit (BD Biosciences), according to the manufacturer's instructions. *Columns*: means; *bars*: SE. Experiments were done in triplicate at three samples per point. Data are from one representative experiment.

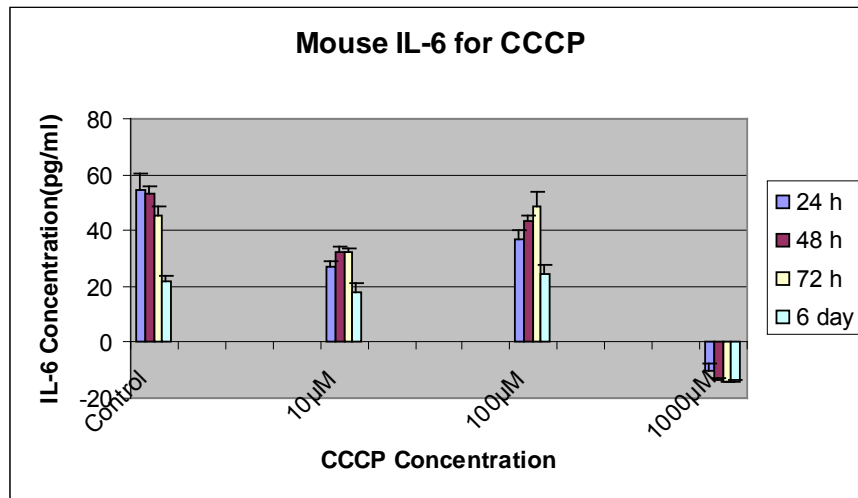


Figure 4. Time and dose effects of CCCP on IL-6 in the media of murine stromal cells. Near confluent stromal monolayers cultured in 24-well plates were treated with CCCP for 1 hour and then washed once with PBS. Cells were then incubated in conditioned medium with only 5% FBS. Supernatant samples were collected at 24h, 48h, 72 hours and 6 days. The IL-6 levels in the supernatant were determined using an IL-6 ELISA kit (BD Biosciences), according to the manufacturer's instructions. *Columns*: means; *bars*: SE. Experiments were done in triplicate at three samples per point. Data are from one representative experiment.

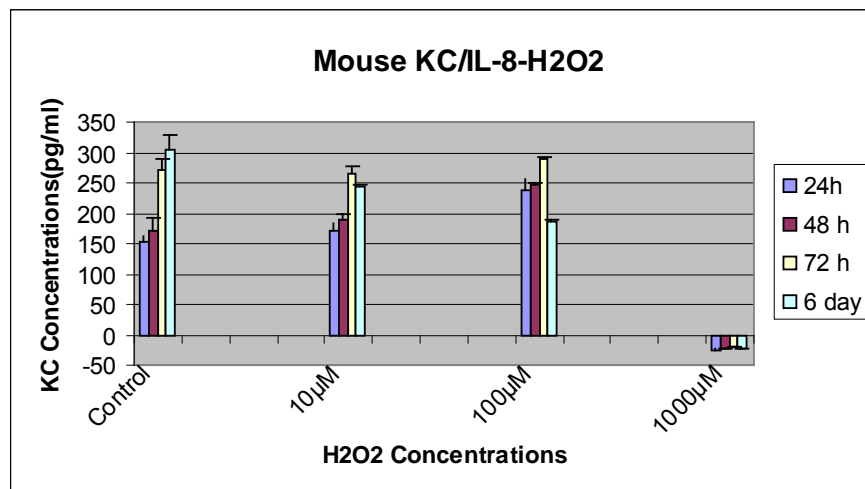


Figure 5. Time and dose effects of H₂O₂ on IL-8 in the media of murine stromal cells. Near confluent stromal monolayers cultured in 24-well plates were treated with H₂O₂ for 1 hour and then washed once with PBS. Cells were then incubated in conditioned medium with only 5% FBS. Supernatant samples were collected at 24h, 48h, 72 hours and 6 days. The IL-8 levels in the supernatant were determined using a murine IL-8 (KC) ELISA kit (R&D Systems), according to the manufacturer's instructions. *Columns*: means; *bars*: SE. Experiments were done in triplicate at three samples per point. Data are from one representative experiment.

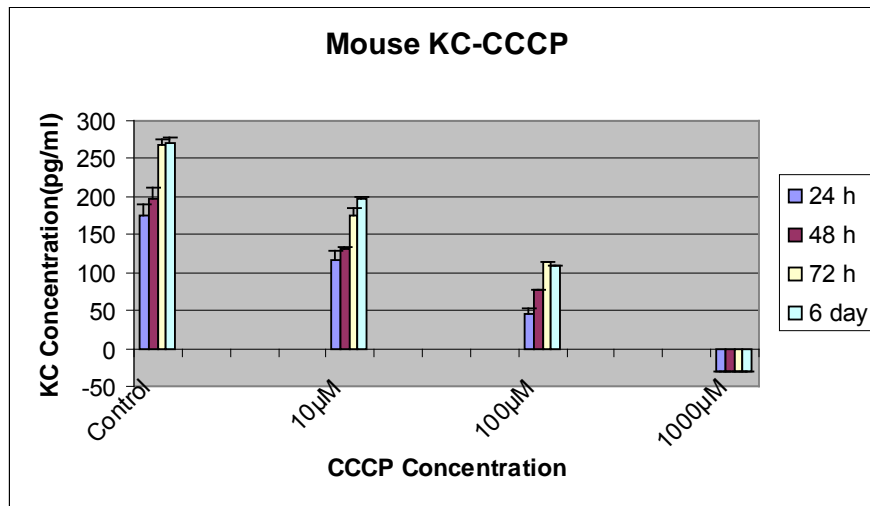


Figure 6. Time and dose effects of CCCP on IL-8 in the media of murine stromal cells. Near confluent stromal monolayers cultured in 24-well plates were treated with CCCP for 1 hour and then washed once with PBS. Cells were then incubated in conditioned medium with only 5% FBS. Supernatant samples were collected at 24h, 48h, 72 hours and 6 days. The IL-8 levels in the supernatant were determined using a murine IL-8 (KC) ELISA kit (R&D Systems), according to the manufacturer's instructions. *Columns*: means; *bars*: SE. Experiments were done in triplicate at three samples per point. Data are from one representative experiment.

IL-6 was significantly higher in stromal conditioned medium ($p < 0.05$, Student's *t* test) 24, 48 and 72 hours after treatment with H_2O_2 10 and 100 μM . The effect ebbed after 6 days but remained significantly higher with the 100 μM treatment. Treatment with 1,000 μM H_2O_2 did not result in IL-6 secretion, likely due to outright rapid toxin-induced cell death. CCCP treatment did not result in an increase in IL-6 export to the conditioned media under any conditions assayed in mouse stroma.

Conditions for induction of IL-8 in mouse stroma differed slightly from those for IL-6 induction. Prolonged incubation of near confluent stroma from 3 to 7 days spontaneously induced significant increases in IL-8 export. After H_2O_2 transient treatment, IL-8 export to the media increased significantly from that of untreated stroma only after treatment with 100 μM H_2O_2 and only during the first two days compared with controls. Treatment with 1,000 μM H_2O_2 resulted in an immediate decrease in IL-8 in the conditioned media, sustained for 7 days, likely due to the immediate death of the cells. As with H_2O_2 , CCCP treatment also did not result in an increase in IL-8 in the conditioned media over control levels.

We carried out western blots to determine whether hypoxic and oxidative injury resulted in activation of the TGF beta pathway. Figure 7 demonstrates that both CCCP and H₂O₂ induced phosphorylation of SMAD-3 as a reflection of TGFβ activation. The effect of H₂O₂ appeared to be more intense. These experiments are ongoing.

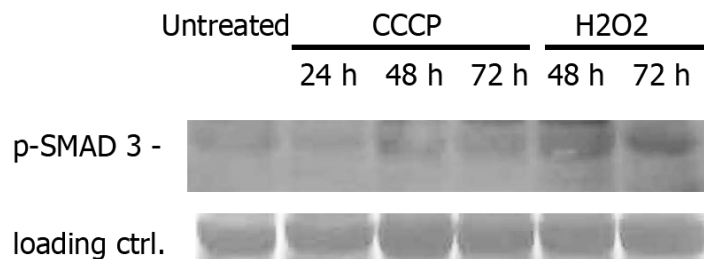


Figure 7. Western blot for phospho-SMAD3 of mouse stromal cells treated with H₂O₂ and CCCP. Nearly confluent mouse stromal monolayers maintained in 10cm plastic dishes were treated with CCCP and H₂O₂ 100 μ M for one hour, washed with PBS and incubated in DMEM 5% FCS for 24, 48 or 72 hours. Cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology) and analyzed by SDS-PAGE. Membranes were probed with antibodies to mouse IL-6 (Abcam Inc.), Cox-2(Cell Signaling), TGF-β(Cell Signaling), phospho-SMAD-2 (Ser 465/467), total SMAD-2, phospho- SMAD-3 (Ser 423/425) and total SMAD-3 (Cell Signaling). Uniform bands from Coomassie Blue-stained membranes were used to verify equal loading. Shown is a membrane demonstrating increased phospho- SMAD-3 staining two and three days after treatments with hypoxic and oxidative stimuli. Other western blots with the other listed antibodies and controls are ongoing.

Once control studies with hypoxic and oxidative injury collected sufficient data to identify doses and times after initial incubation that resulted in quantifiable injury and evidence of secretory senescence in mouse stromal cells, we proceeded to determine if estrogen deprivation induced secretory senescence in stroma. We assessed IL-6 and IL-8 secretion and TGFβ signaling as indicators of secretory senescence. Nearly confluent stromal monolayers were incubated without and with ICI182780, and estrogen receptor alpha (ERα) inhibitor at variable concentrations of 10⁻⁸, 10⁻⁷ and 10⁻⁶ M for 1, 2, 3, 4, 7, 13, 21 and 28 days in phenol red-free medium. We used 100 μ M H₂O₂ incubation and IL-6 secretion after 48 hours as a positive control. Figure 8 demonstrates that deprivation of estrogen induces export of IL- 6 by mouse stroma into the medium after 14 hours. The level of IL-6 peaks after 72 hours, then abates. However, levels remain measurable and significantly higher than those in untreated stroma for the entire four weeks.

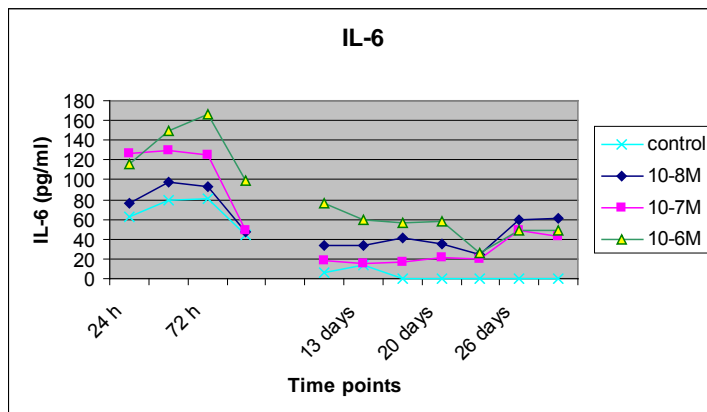


Figure 8. Time and dose effects of ICI182780 on IL-6 in the medium of murine stromal cells. Near confluent stromal monolayers were cultured in 24-well plates in phenol red-free DMEM 10% fetal calf serum with ICI182780 at variable concentrations of 10⁻⁸, 10⁻⁷ and 10⁻⁶ M for variable times until 4 weeks. Medium and ICI were replenished twice a week. Supernatant samples were collected at 1, 2, 3, 4, 7, 13, 21 and 28 days. The IL-6 levels in the supernatant were determined using an IL-6 ELISA kit (BD Biosciences), according to the manufacturer's instructions. *Columns*: means; *bars*: SE. Experiments were done in triplicate at three samples per point. Data are from one representative experiment.

To determine the effect of estrogen deprivation on activation of the TGF β pathway we carried out western blots to determine the phosphorylation of SMAD-2 in lysates from stroma deprived of estrogen stimulations. Figure 9 demonstrates that murine stroma incubated with variable concentrations of ICI182780 for 24 and 48 hours in the absence of phenol red induced phosphorylation of phospho-SMAD2, indicative of TGF β signal pathway activation.

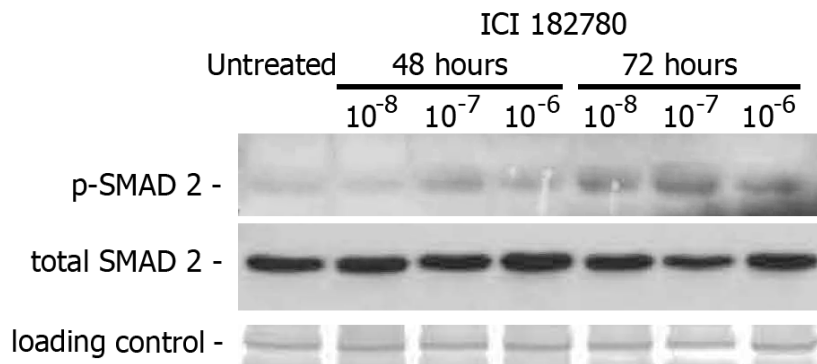


Figure 9. Western blot for phospho-SMAD2 of mouse stromal cells treated with estrogen deprivation. Nearly confluent mouse stromal monolayers maintained in 10 cm plastic dishes were treated with ICI 182780 of 10⁻⁸, 10⁻⁷ and 10⁻⁶ M for 48 hr and 72 hr were lysed in modified RIPA buffer and analyzed by SDS-PAGE with antibody to mouse phospho-SMAD-2 (Ser 465/467) and total SMAD-2 (Cell Signaling). Uniform bands from Coomassie Blue-stained membranes were used to verify equal loading. The data demonstrate increased phospho- SMAD2 staining with two and three days of estrogen deprivation. Other western blots with antibodies and controls querying TGF beta signaling and COX 2 activation are ongoing.

We carried out several controls to demonstrate that ICI182780 at the doses used was effective. Kinetic experiments with MCF-7 estrogen receptor positive and MDA-MB-231 estrogen receptor negative breast cancer cell lines demonstrated that ICI182780 at 10⁻⁸ M was sufficient to inhibit the proliferation of MCF-7 cells, while, as a negative control, concentrations as high as 10⁻⁶ M had no effect on the proliferative rate of MDA-MB-231 cells (Figure 10). Western blots of lysates prepared from MCF-7 cells incubated with variable concentrations of ICI182780 for 48 and 72 hours demonstrated the reactive upregulation of ER α in these cells in response to estrogen blockade (Figure 11).

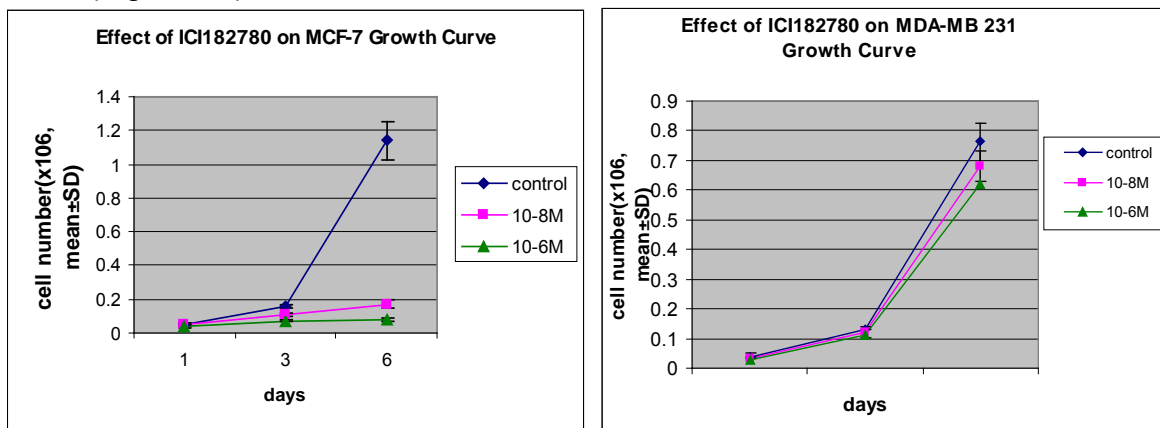


Figure 10. Effect of ICI182780 on MCF-7 and MDA-MB231 cell growth. A total of 5x10⁴ breast cancer cells were cultured in 24 well plates in triplicate wells for 1, 2 and 6 days with ICI182780 was added at the time of initial incubation. Cell number in each well was counted using cell counter. Bars: SD.

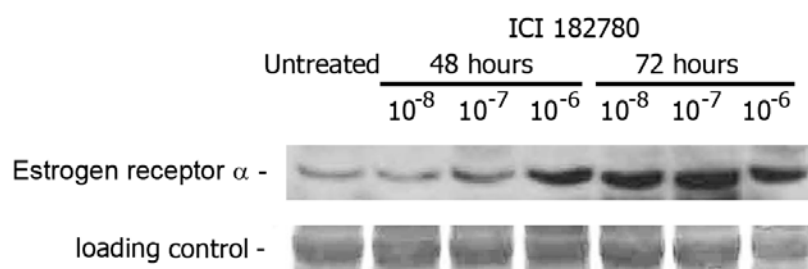


Figure 11. Western blot for ER α in MCF-7 cells treated with estrogen inhibitor ICI182780. Semiconfluent MCF-7 cells maintained in 10 cm plastic dishes were treated with a series of concentrations of ICI 182780 of 10^{-8} , 10^{-7} and 10^{-6} M for 48 hr and 72 hr. Cells were then lysed in modified radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology) and analyzed by SDS-PAGE. Membranes were probed with antibody human ER α (Santa Cruz Biotechnology). Coomassie Blue-stained membranes were used to verify equal loading.

This set of experiments with murine stroma permitted us to establish conditions, assays, time and dose ranges for inducing and quantitating secretory senescence in stroma. We began collecting human bone marrow from normal volunteers under a UMDNJ and US Army IRB-approved protocol. The personnel conducting these assays only joined the lab in January and the presentation of volunteers was very sparse initially. We collected bone marrow aspirates from two pre-menopausal volunteers and established stromal cultures from their samples using standard procedures. The aspirate was centrifuged at 1,500 rpm for 15 minutes and buffy coat was collected and cultured in a 25cm² flask in complete McCoy's medium. Several important differences became apparent between the conditions and behavior of murine and human stroma. The first difference is that human stroma grow at a far slower rate than murine stroma. In contrast to three weeks needed for a confluent 25 cm² flask to be near confluent from the femurs of 3 mice, human stroma obtained from 20 ml of bone marrow aspirate required 8 weeks to be sufficiently confluent to permit passage to 24 well plates. After 8 weeks the cells were trypsinized and distributed onto all of the wells of a 24 well plate and permitted to grow to near confluence for about 2 weeks.

Confluent monolayers of human stromal cells on 24 well plates were treated with H₂O₂, CCCP and ICI182780 in quadruplicate. Rows of 4 wells were either untreated or treated with 100 μ M H₂O₂, 100 μ M CCCP for 1 hr at 37 $^{\circ}$, washed with PBS and then replenishes with McCoy's complete growth medium. To the fourth row, 10^{-6} M ICI182780 in phenol red-free medium was added, in which cells were maintained throughout the duration of the experiment. Supernatants from each well were collected at specified time points and stored at -80 $^{\circ}$. ELISAs were performed on thawed conditioned media for IL-6 and IL-8 using a Human IL-6 Elisa Kit from BD & Human CXCL8/IL-8 kit from R&D Systems respectively.

Figure 12 demonstrates that statistically significant differences in IL-6 export between control and CCCP-treated cells occurred during most of the time points from 1 to 28 days, suggesting a more sensitive response to hypoxia by human stroma than by murine stroma. It also demonstrated statistically significant differences in IL-6 export between control and estrogen deprived stroma after a week of incubation that was sustained for the 28 days assayed. This also suggests that estrogen deprivation initiates a sustained secretory pattern in human stroma at levels that relatively much higher than those in murine stroma. These data will need to be confirmed in numerous other donor volunteers, as outlined by the statistical section of our grant application. Extensive discussions and meetings with our statistician collaborator have reconciled our initial plan and the plan for data analysis, depending on whether the donor sample data distribute in a normal distribution or whether they do not, in which case transformation of the data

and non parametric analysis will need to be used. In addition, we will need to analyze human and murine stroma side by side on the same dishes to compare values and confirm the suggested inferences.

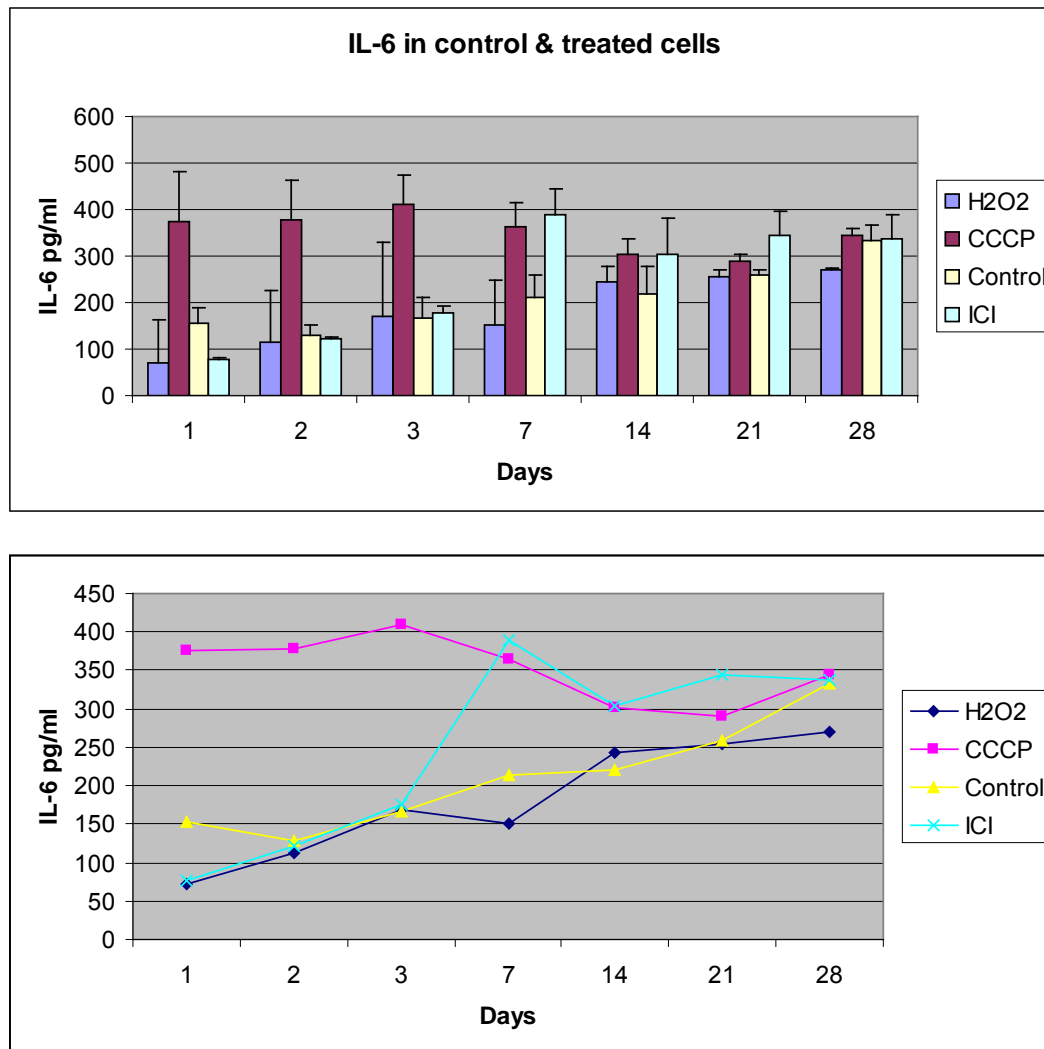


Figure 12. ELISA assay for IL-6 in a pre-menopausal human female stromal monolayers treated with oxidative, hypoxic and estrogen deprivation injury for variable time points. Quadruplicate wells of confluent monolayers of human stromal cells on 24 well plates were treated with 100 μ M H₂O₂, 100 μ M H₂O₂ CCCP for one hour, washed with PBS and then replenishes with McCoy's complete growth medium and incubated for variable times from 1 to 28 days, or treated and maintained in 10⁻⁶ M ICI182780 in phenol red-free medium throughout the duration of the experiment from 1 to 28 days. Supernatants from each well were collected at specified time points and stored at -80°. ELISAs were performed of thawed conditioned media for IL-6 using a Human IL-6 Elisa Kit from BD. The bar graph represents the data and standard deviations, demonstrating statistically significant differences in IL-6 export between control and CCCP-treated cells for most of the time points and statistically significant differences between IL-6 export between control and estrogen deprived stroma after a week of incubation. The lower line graph is included to better visually demonstrate these trends. No impact was demonstrated by oxidative injury on IL-6 export.

Figure 13 demonstrates that IL-8 was not increased in human stroma as a consequence of incubations with either H₂O₂, CCCP or estrogen deprivation up until day 7. Similarly, Figure 14 demonstrates that estrogen deprivation to 28 days also did not induce IL-8 secretion by human stromal monolayers.

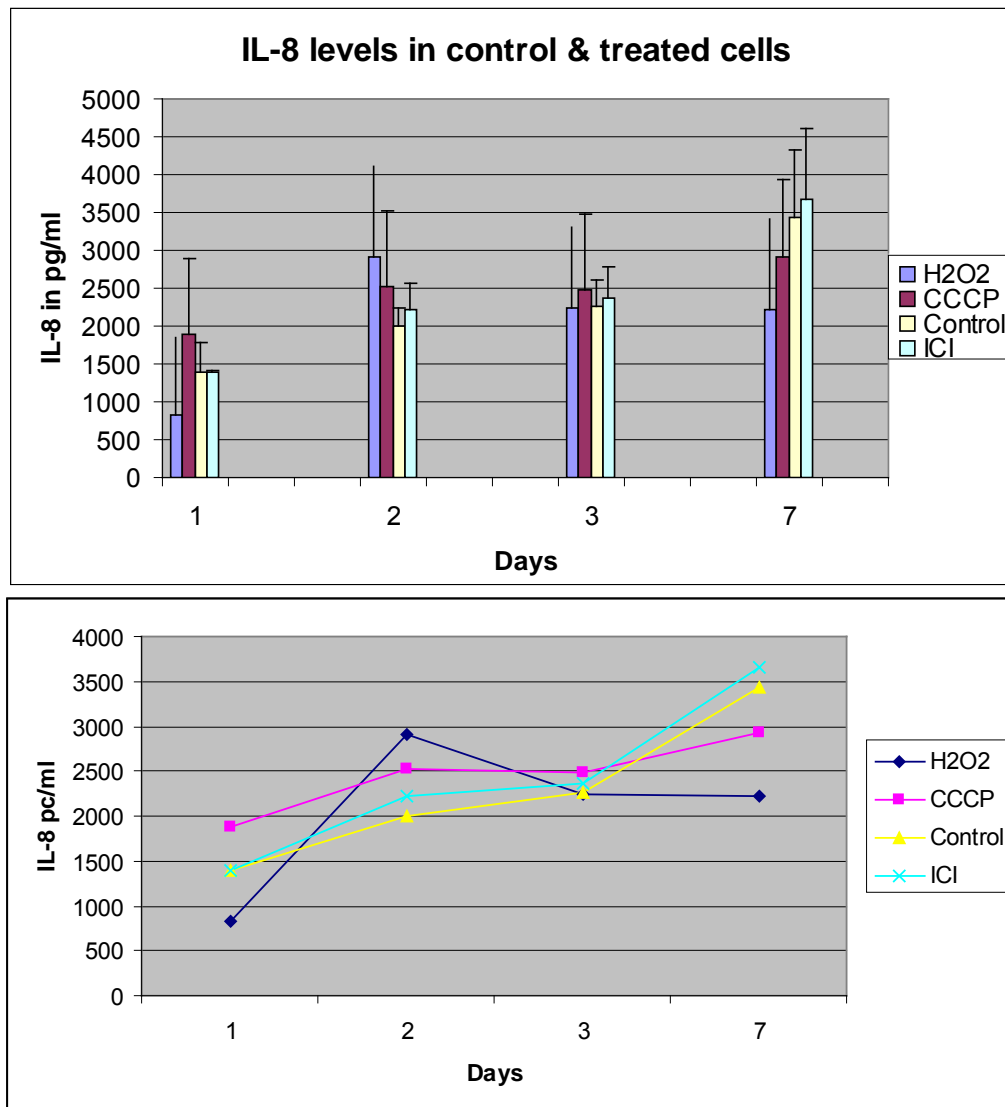


Figure 13. ELISA assay for IL-8 in a pre-menopausal human female stromal monolayers treated with oxidative, hypoxic and estrogen deprivation injury for variable time points. Quadruplicate wells of confluent monolayers of human stromal cells on 24 well plates were treated with 100 μ M H₂O₂, 100 μ M H₂O₂ CCCP for one hour, washed with PBS and then replenishes with McCoy's complete growth medium and incubated for variable times from 1 to 7 days, or treated and maintained in 10⁻⁶ M ICI182780 in phenol red-free medium throughout the duration of the experiment from 1 to 7 days. Supernatants from each well were collected at specified time points and stored at -80°. ELISAs were performed of thawed conditioned media for IL-6 using a Human CXCL8/IL-8 kit from R&D Systems. The bar graph represents the data and standard deviations, demonstrating no statistically significant increases in IL-8 export between experimental and control values at all in CCCP, H₂O₂ and ICI182780 treated cells. The lower line graph is included for better visual depiction.

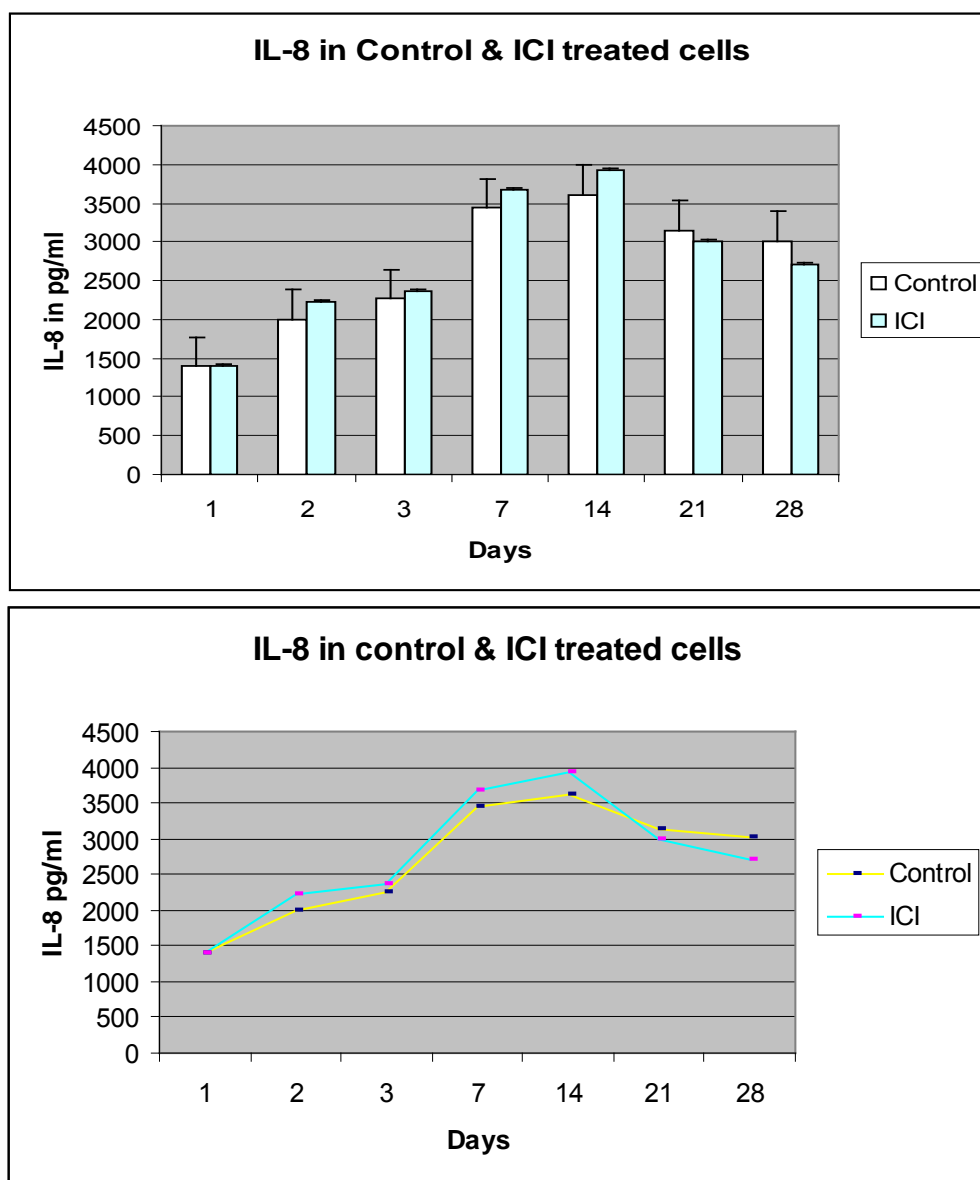


Figure 14. ELIZA assay for IL-8 in a pre-menopausal human female stromal monolayers treated with estrogen deprivation injury for variable time points until 28 days. Quadruplicate wells of confluent monolayers of human stromal cells on 24 well plates were treated and maintained in 10^{-6} M ICI182780 in phenol red-free medium throughout the duration of the experiment from 1 to 28 days. Supernatants from each well were collected at specified time points and stored at -80° . ELISAs were performed of thawed conditioned media for IL-6 using a Human CXCL8/IL-8 kit from R&D Systems. The bar graph represents the data and standard deviations, demonstrating no statistically significant increases in IL-8 export between experimental and control values. The lower line graph is included for better visual depiction.

Our goal is to determine if estrogen deprivation-induced stromal secretory senescence deprives bone marrow stroma of its capacity to support dormant colonies in co-culture. The dormancy model we will use involves three components: estrogen sensitive breast cancer cells, partial redifferentiation with FGF-2 that results in re-expression of integrins lost with malignant transformation and fibronectin, a component of bone marrow stroma that initiates specific survival signaling through PI3K when it ligates newly re-expressed integrin $\alpha 5 \beta 1$ on breast cancer cells.

Figure 15 demonstrates our standard clonogenic assays for dormant colony formation of MCF-7 cells incubated on fibronectin-coated tissue culture plates with FGF-2. The clonogenic potential to form >30 cell growing clones is disrupted by incubation with FGF-2 and the formation of 2-12 cell dormant clones consisting of enlarged, flattened cells with large cytoplasm to nucleus ratios with cortically rearranged f-actin and inactivated RhoA is enabled (Korah, et al., 2004; Barrios and Wieder, 2009). We repeated the assay in preparation for co-incubating these cells with human stromal monolayers before and after estrogen-deprivation-induced secretory senescence.

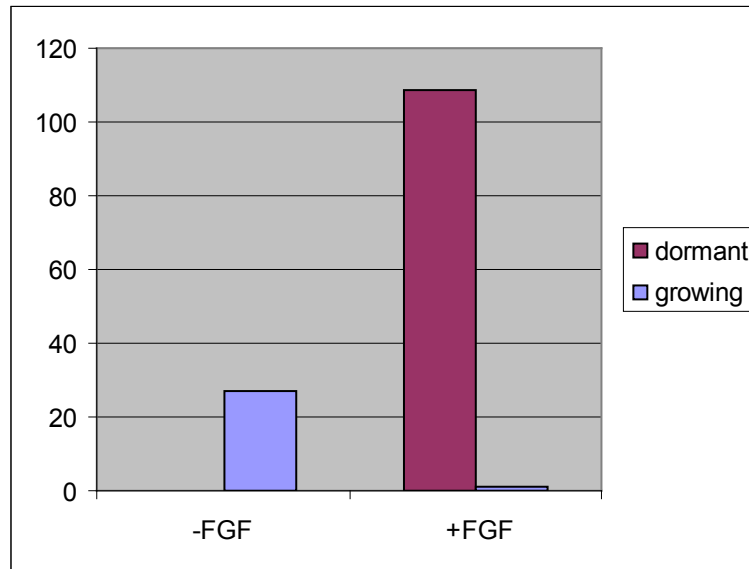


Figure 15. Formation of dormant clones by MCF-7 on Fibronectin with FGF-2. A total of 1,000 MCF-7 cells were incubated on Fibronectin-coated 24-well plates in quadruplicate with and without 10 ng/ml FGF-2 that was added the following day. Six days after FGF-2 addition, growing colonies of >30 cells and dormant clones of 2-12 cells with the distinct appearance of large, spread cells with large cytoplasmic to nucleus ratios were counted. The data demonstrate almost complete disappearance of growing clones and the appearance of dormant clones in the FGF-2 treated wells.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed *in vitro* stromal injury model in mouse bone marrow stroma
- Developed *in vitro* stromal injury model in human bone marrow stroma
- Defined time and dose parameters for H₂O₂-induced IL-6 and IL-8 export by murine stroma
- Defined time and dose parameters for H₂O₂-induced IL-6 export by human stroma
- Defined time and dose parameters for estrogen deprivation-induced IL-6 export by murine and human stroma
- Demonstrated TGFβ pathway activation by hypoxic, oxidative and estrogen deprivation-induced injury in bone marrow stroma

REPORTABLE OUTCOMES:

The data has not been presented in any forum yet at this time.

CONCLUSION:

The data demonstrate, for the first time, that bone marrow stroma develop characteristics described in the state called secretory senescence. This cellular condition has been described in other systems and consist of an injured state that, among other traits, is associated with inflammatory signaling, activation of the TGF β pathway and secretion of inflammatory cytokines. We hypothesized that estrogen deprivation may be one mechanism that can induce secretory senescence in the bone marrow stroma, and effect that may be universal in menopause. This is potentially of great significance, as the secreted inflammatory cytokines may induce breast cancer micrometastases that have been dormant in the stromal microenvironment for extended periods to begin proliferating again and result in incurable recurrent disease.

We tested the hypothesis that estrogen deprivation induces secretory senescence in both murine and human stroma and demonstrated that these stromal cells begin to secrete IL-6 and activate TGF β signaling in response to estrogen deprivation. We will continue to conduct experiments to support these observations and will follow up with studies to determine if this inflammatory state results in the loss of stromal capacity to support dormancy. We will also determine if post-menopausal women have a baseline secretory senescence component to their stroma and whether this condition can be reversed by estrogen restoration *in vitro* and by anti-inflammatory treatment.

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Korah R, Boots M, and Wieder R. (2004) Integrin $\alpha 5\beta 1$ promotes survival of growth-arrested breast cancer cells: an *in vitro* paradigm for breast cancer dormancy in bone marrow. *Cancer Research* 64: 4514-4522.

Barrios, J and Wieder R. (2009) Dual FGF-2 and intergrin $\alpha 5\beta 1$ signaling mediate GRAF-induced RhoA inactivation in a model of breast cancer dormancy. *Cancer Microenvironment* 2:33-47.

APPENDICES:

None

SUPPORTING DATA:

All data appear in the body.